p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells

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The finding of frequent nitric oxide synthase expression in human cancers indicates that nitric oxide has a pathophysiological role in carcinogenesis. To determine the role of nitric oxide in tumor progression, we generated human carcinoma cell lines that produced nitric oxide constitutively. Cancer cells expressing inducible nitric oxide synthase that had wild-type p53 had reduced tumor growth in athymic nude mice, whereas those with mutated p53 had accelerated tumor growth associated with increased vascular endothelial growth factor expression and neovascularization. Our data indicate that tumor-associated nitric oxide production may promote cancer progression by providing a selective growth advantage to tumor cells with mutant p53, and that inhibitors of inducible nitric oxide synthase may have therapeutic activity in these tumors.

Increased expression of inducible nitric oxide synthase (NOS2) has been found in a variety of human cancers[1–4], and a NOS2-specific inhibitor can reduce growth of xenografted tumors in mice[5]. Hypoxia upregulates NOS2 expression[6], and nitric oxide (NO) induces mitogenesis among endothelial cells[7]. NO induces vascular endothelial growth factor (VEGF) expression in carcinoma cells[8] and neovascularization in tumors[4,9]. Thus, the promotion of tumor growth by NO[9,10] may involve the induction of angiogenic factors[11]. However, the function of NO in carcinogenesis is uncertain. NO has been found to either inhibit[12] or stimulate[9,10] tumor growth. High concentrations of NO are also known to induce cell death in many cell types, including tumor cells[13–15], whereas lower concentrations of NO can have an opposite effect and protect against apoptotic cell death from various stimuli[16,17]. We investigated the role of NO in tumor growth using carcinoma cells genetically engineered to produce NO constitutively, and found that the effect of NO on tumor growth is p53-dependent and that endogenously produced NO accelerates tumor growth of mutant p53 cells by inducing VEGF and neovascularization.

Constitutive expression of NOS2 in human carcinoma cell lines

High concentrations of NO induce p53 accumulation and p53-mediated growth arrest or apoptosis[18,19]. To investigate the functional interaction of p53 and NO in tumor growth, we infected human carcinoma cells, which had either a wild-type p53, missense mutant p53 or p53-null status, with a retroviral construct, DFG-iNOS (ref. 20). The amounts of NO produced by 10^6 of these cells ranged from 5 to 15 nmole of nitrite plus nitrate per day (Table), which is substantially lower than NO production in cytokine-stimulated macrophages[21]. Isogenic vector-control carcinoma cell lines, which expressed β-galactosidase (β-gal) instead of human NOS2 (ref. 20), did not produce detectable amounts of NO.

NOS2 expression and tumor growth

We investigated the effects of NOS2 expression on the growth rates of human carcinoma cells both in cell culture and in subcutaneous tumors in athymic nude mice. In cell culture, NOS2-expressing carcinoma cell clones grew at the same rate as isogenic vector controls (Fig. 1*a* and *b*). Though NO cytotoxicity has been described in tumor cells after transfection with murine NOS2[9,12], it was not observed in the cell clones infected with DFG-iNOS, which is consistent with the moderate NO production in these cell lines. To further evaluate whether NO alters tumor growth, we inoculated carcinoma cells expressing either NOS2 or β-gal subcutaneously into athymic nude mice and

Table Nitric oxide production[a] in human carcinoma cell lines constitutively expressing NOS2

Cell line			Nitrite plus nitrate nmole/day/ per 1×10^6 cells
Calu-6	BaglacZ		ND
	NOS2	Clone 5	8
	NOS2	Clone 7	11
LoVo	BaglacZ		ND
	NOS2	Clone 9	6
RKO	BaglacZ		ND
	NOS2	Clone 5	6
HCT-116	BaglacZ		ND
	NOS2	Clone 1	2
	NOS2	Clone 2	3
	NOS2	Clone 3	4
HT-29	BaglacZ		ND
	NOS2	Clone 1	3
	NOS2	Clone 2	8
	NOS2	Clone 3	15

[a]Nitrite plus nitrate accumulation in the cell culture medium
ND, not detectable

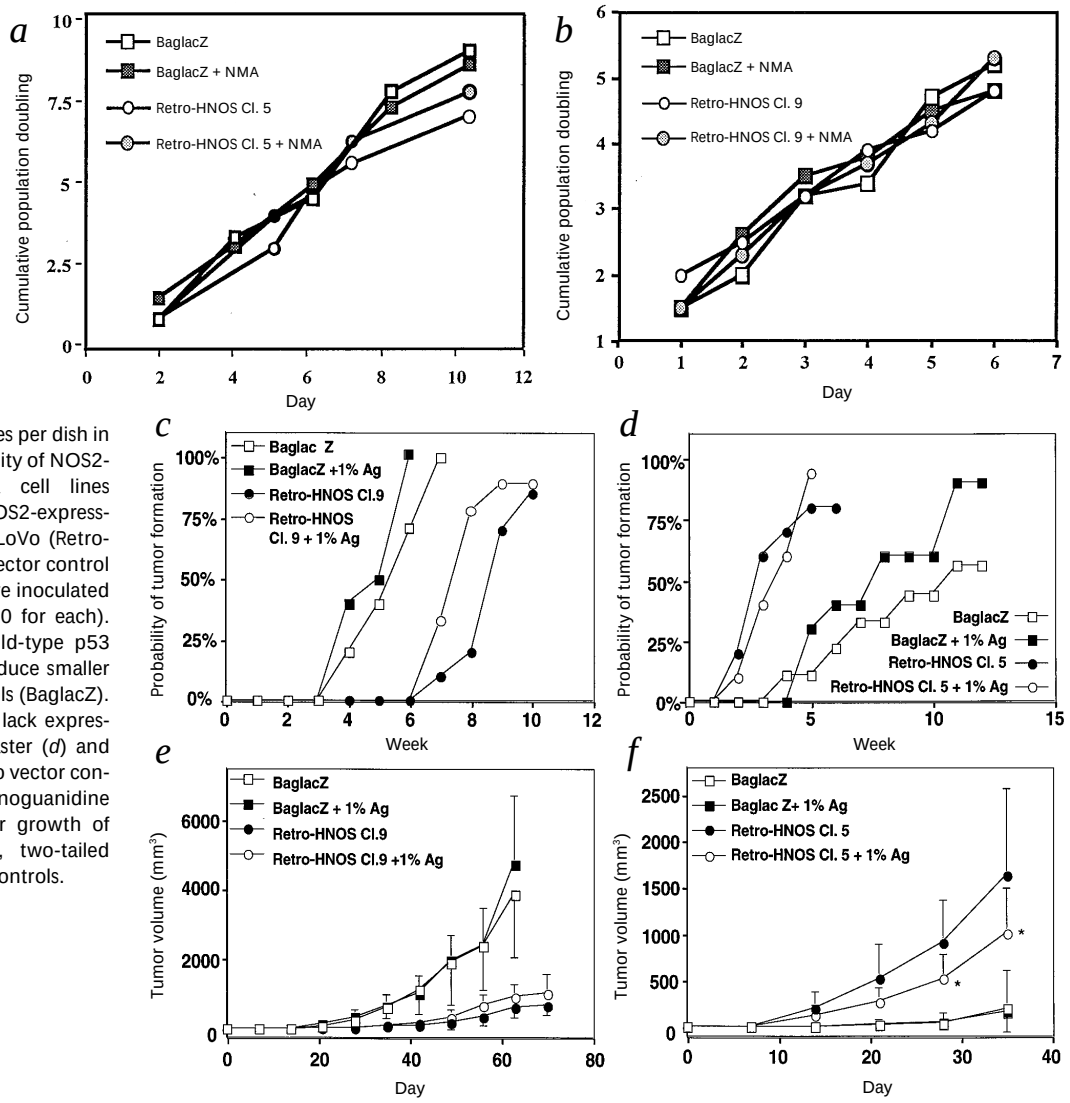

Fig. 1 NOS2 expression and tumor growth. ***a*** and ***b***, NO production in human carcinoma cells does not change cell growth in cell culture. Retro-HNOS-expressing Calu-6 (Retro-HNOS Cl.5) and LoVo (Retro-HNOS Cl.9) carcinoma cells were cultured in the presence or absence of 2 mM of the NOS inhibitor N^G-monomethyl-L-arginine (NMA). Clonal cell growth was compared with that of vector controls (BaglacZ). Each point represents the average clonal growth of 10 colonies per dish in three dishes. ***c***–***f***, Tumor probability of NOS2-expressing human carcinoma cell lines depends on their p53 status. NOS2-expressing Calu-6 (Retro-HNOS Cl.5), LoVo (Retro-HNOS Cl.9) carcinoma cells or vector control cells (BaglacZ) (3×10^6 cells) were inoculated into athymic nude mice (n = 10 for each). LoVo cells, which have two wild-type p53 alleles, grow slower (*c*) and produce smaller tumors (*e*) than do vector controls (BaglacZ). In contrast, Calu-6 cells, which lack expression of functional p53, grow faster (*d*) and produce larger tumors (*f*) than do vector controls. The NOS2 inhibitor aminoguanidine (1% AG) suppressed the tumor growth of Calu-6 cells (*f*; *, $P < 0.05$, two-tailed Student's *t*-test) but not vector controls.

monitored tumor growth. NO-producing LoVo cells that expressed wild-type p53 grew slower and produced smaller tumors than did isogenic vector controls (Fig. 1*c* and *e*). Analysis of tumor extracts demonstrated that protein concentrations of the cell-cycle inhibitor $p21^{WAF1}$, a downstream target of p53 (ref. 22), was significantly ($P < 0.01$, two-tailed Student's *t*-test) higher in tumors of NOS2-expressing LoVo cells than in vector controls (Fig. 2). Pro-apoptotic cleavage of poly(ADP-ribose)polymerase (PARP) was not detected (data not shown). In contrast to LoVo cells, NO-producing Calu-6 cells, which are p53-null, grew faster and produced larger tumors than did isogenic vector control cells (Fig. 1*d* and *f*).

The observation that NO affects tumor growth depending on the p53 status was extended by additional studies. NO affected tumor growth in a dose-dependent manner (Fig. 3*b*), and also reduced the tumor growth of two other colon carcinoma cell lines with wild-type p53 (RKO and HCT-116 cells), whereas it accelerated the growth of a colon carcinoma cell line homozygous for mutant p53 (codon 273^{His}; HT-29 cells)(Fig. 3*a*). The tumors derived from NOS2-expressing cell lines had NOS2 activities similar to those frequently found in a cohort of colorectal tumors[3] and ranged from 3 to 25 pmole/min/mg. Furthermore, aminoguanidine, a specific inhibitor of NOS2 (ref. 23) significantly reduced the tumor growth of NOS2-expressing Calu-6 ($P < 0.05$, two-tailed Student's *t*-test; Fig. 1*d* and *f*) and HT-29 cells ($P = 0.002$, Kaplan-Meier analysis; Fig. 3*a*).

NO-induced neovascularization

We next investigated mechanisms whereby endogenous NO production could accelerate the tumor growth of carcinoma cells that are either null or mutant for p53. NO has angiogenic properties and has been shown to increase blood vessel density in tumors grown by DLD-1 human colon carcinoma cells transfected with murine NOS2 (ref. 9). Therefore, we analyzed subcutaneous tumors produced by Calu-6 cells in nude mice for angiogenesis by doing immunohistochemistry for CD31, which is a specific marker of endothelial cells and vascularization[24]. We found that tumors expressing NOS2 contained significantly ($P < 0.01$, two-tailed Student's *t*-test) more small blood vessels than did tumors lacking NOS2 (Fig. 4). Vector control tumors had large necrotic areas not found in tumors expressing NOS2; perhaps deficient angiogenesis limited the growth of these controls. Our observations are consistent with reports linking endogenous NO production to an increased tumor growth rate, presumably by enhancing angiogenesis[9]. Based on these observations, the lack of an aminoguanidine effect in slow-growing

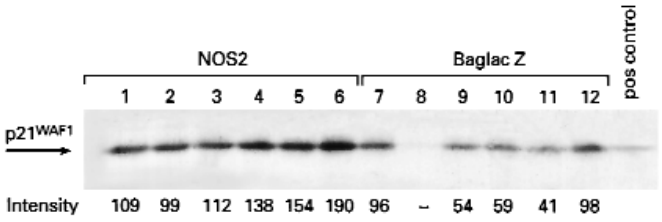

Fig. 2 Expression of p21^{WAF1} protein is higher in tumors of NOS2-expressing LoVo cells than in tumors of the vector controls (BaglacZ). Western blot analysis of p21^{WAF1} protein concentrations in 12 tumor extracts. The average p21^{WAF1} protein concentration in tumors of vector controls is 43% that of tumors of NOS2-expressing LoVo cells (58 ± 37 compared with 134 ± 34; $P < 0.01$, two-tailed Student's *t*-test). A nuclear extract provided with the anti-p21^{WAF1} antibody was used as positive control (far right lane).

tumors of NOS2-expressing LoVo cells might be explained by insufficient microvascularization; that is, not allowing an effective inhibitor concentration, whereas the more vascular tumors of NOS2-expressing Calu-6 cells were inhibited by the higher concentration of aminoguanidine.

VEGF expression and apoptosis in NOS2-expressing cells

To explore the angiogenic activity of NO, we investigated VEGF as a downstream effector. NO is capable of depleting the intracellular iron storage, thereby activating the IRE binding protein[25]. Iron depletion also activates VEGF expression[26]. Therefore, we investigated VEGF mRNA and protein expression in carcinoma cells expressing NOS2. VEGF protein concentrations were higher in cellular extracts of NOS-expressing clones than in extracts of the vector control cell lines (Fig. 5*a*). To further buttress this finding, we determined VEGF mRNA levels in Calu-6 cells. VEGF mRNA steady-state concentrations were increased in two cell clones expressing NOS2 compared with those of the β-gal-expressing vector controls (Fig. 5*c*). The VEGF mRNA expression levels also correlated with an increased secretion of VEGF protein into the culture medium (Fig. 5*b*). The addition of an NOS inhibitor, N^G-monomethyl-L-arginine (L-NMA), to the cell culture medium reduced the VEGF secretion (Fig. 5*b*). These results demonstrate that endogenously produced NO increases VEGF secretion in human carcinoma cells, which is consistent with a report showing that NO donors induce guanylate cyclase-dependent upregulation of VEGF mRNA (ref. 8). Increased VEGF mRNA levels were also found in tumors of NOS2-expressing Calu-6 and HT-29 carcinoma cells. The level of VEGF mRNA, calculated per copy of β-actin mRNA, correlated positively with NOS2 activity in the HT-29 clones, and increased from 0.25 in tumor extracts of vector controls to 0.31 in HT-29 clone 1, 0.51 in HT-29 clone 2 to 0.56 in HT-29 clone 3, which has the highest NOS2 activity (Table). VEGF mRNA levels varied in tumors of wild-type p53 carcinoma cells, and a correlation between NOS2 activity and VEGF expression was not found for tumors of HCT-116 cells. Because moderate NO production inhibits apoptosis from various stimuli, we tested whether endogenous NO production inhibits pro-apoptotic cleavage of PARP (ref. 16) in HT-29 cells. We found that NO is an inhibitor of PARP cleavage (Fig. 6) and activation of caspases (data not shown) in sulindac-induced apoptosis[27] of HT-29 cells. Thus, the moderate NO production may increase both the vascularization of tumors and the survival rate of cells in a tumor environment.

Discussion

We report here that endogenously produced NO inhibits growth of tumor cells with wild-type but not mutant p53 in the athymic nude mouse model, and induces VEGF expression and angiogenesis in tumors with mutant p53. A reciprocal relation between VEGF and NO has been observed with an angioplasty model[28]. We found an opposite relationship in human tumor cells, and confirm data showing that NO released by an NO-donor upregulates VEGF mRNA levels in human carcinoma cells[8]. Upregulation of VEGF by NO coincided with increased vascularization in xenografted tumors of NOS2-expressing Calu-6 and HT-29 cells. This correlation between NOS2 expression and tumor vascularization has also been observed with xenografts of

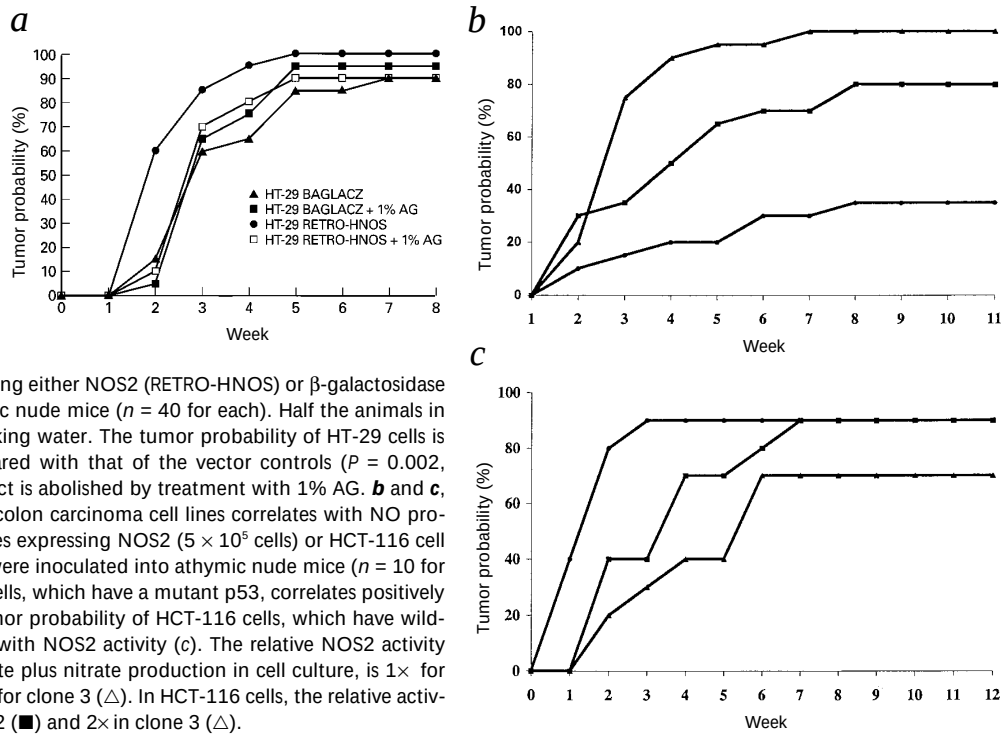

Fig. 3 ***a***, The NOS2 inhibitor aminoguanidine (1% AG) reverses the growth stimulatory effect of NOS2 in tumors of HT-29 colon carcinoma cells. HT-29 cells (3×10^5) expressing either NOS2 (RETRO-HNOS) or β-galactosidase (BAGLACZ) were inoculated into athymic nude mice ($n = 40$ for each). Half the animals in each group received 1% AG in the drinking water. The tumor probability of HT-29 cells is significantly increased by NOS2 compared with that of the vector controls ($P = 0.002$, Kaplan-Meier survival analysis). This effect is abolished by treatment with 1% AG. ***b*** and ***c***, Tumor probability of NOS2-expressing colon carcinoma cell lines correlates with NO production and p53 status. HT-29 cell clones expressing NOS2 (5×10^5 cells) or HCT-116 cell clones expressing NOS2 (1×10^6 cells) were inoculated into athymic nude mice ($n = 10$ for each). The tumor probability of HT-29 cells, which have a mutant p53, correlates positively with NOS2 activity (*b*), whereas the tumor probability of HCT-116 cells, which have wild-type p53, shows an inverse correlation with NOS2 activity (*c*). The relative NOS2 activity (Table) in HT-29 cells, measured as nitrite plus nitrate production in cell culture, is 1× for clone 1 (●), 2.7× for clone 2 (■) and 5× for clone 3 (△). In HCT-116 cells, the relative activities are 1× in clone 1 (●), 1.5× in clone 2 (■) and 2× in clone 3 (△).

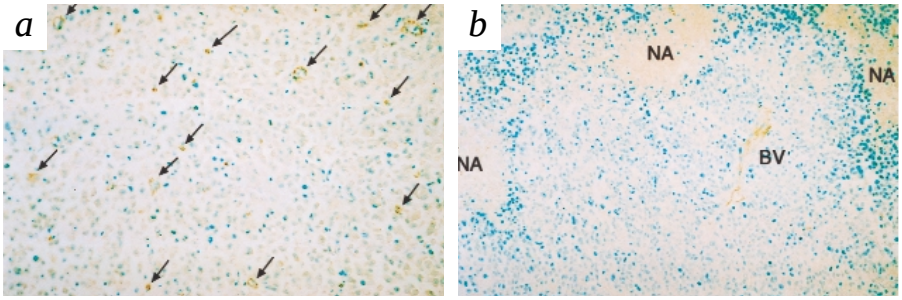

Fig. 4 NO induces tumor microvascularization. Immunohistochemical analysis of the endothelial cell antigen CD31 in tumors grown by Calu-6 lung carcinoma cells expressing NOS2 (*a*) or β-galactosidase (*b*) in athymic nude mice. ***a***, Many capillaries are stained in tumors grown by NOS2-expressing Calu-6 cells (arrows). ***b***, Scanning magnification shows staining of only one longitudinal section of a large blood vessel (BV) in tumors grown by the vector control cells; several necrotic areas (NA) are nearby. CD31-positive microvessels per ×250 field: 6.1 ± 2.8 (NOS2) compared with 0.7 ± 0.7 (vector control); $P < 0.01$, two-tailed Student's *t*-test. Methyl green counterstain. Original magnification ×100.

murine NOS2-transfected DLD-1 human carcinoma cells[9]. Because NOS2 expression in tumor infiltrating monocytes corresponds with the onset of VEGF expression in human colon adenomas[3], and NOS2 is induced by hypoxia[6], induction of NOS2 is probably part of the early response in tumor angiogenesis. The observation that the NOS2-specific inhibitor 1400W limits tumor growth in mice[5] also supports this view.

High levels of NO trigger p53 accumulation[18,19]. We found an increased concentration of $p21^{WAF1}$ in tumors of NOS2-expressing LoVo cells. Induction of $p21^{WAF1}$ is mediated by wild-type p53 and induces growth arrest[22]. Wild-type p53 is a known inhibitor of tumor angiogenesis[29]. Thus, the loss of p53 function in p53-null or p53-mutant cells would permit both the growth of tumor cells in the presence of moderate NO concentrations and the release of angiogenic factors such as VEGF. Increased NOS2 levels have been detected in human breast, brain, head and neck, and colon cancers[1–3], which may lead to a p53-mediated growth arrest in the epithelial cells close to the source of NO production. As our data indicate, the resulting growth inhibition would provide a strong selection pressure for mutant p53. Indeed, breast, brain, head and neck, and colon cancers that can overexpress NOS2[1–4] have a high frequency of p53 mutations[30]. Clonal selection and growth are further supported by NO-induced VEGF expression and angiogenesis. In addition, wild-type p53 transrepresses both basal and cytokine-induced NOS2 in a negative 'feedback loop'[19], so that NOS2 expression would be unchecked in cells with mutant p53. These data are consistent with the hypothesis that NO is a cancer-promoting factor in human carcinogenesis[31].

Methods

Retroviral gene transfer of human NOS2. Human carcinoma cells were infected, as described[20], with either the retroviral vector DFG-iNOS (which has the human NOS2 gene) or with a control vector, BaglacZ (in which NOS2 is replaced with the β-galactosidase gene). Cell clones that constitutively produced nitric oxide were isolated after 14 days of G418 selection (250–350 μg G418 per ml). HCT-116, HT-29, LoVo, RKO colon carcinoma cells, and Calu-6 lung carcinoma cells (all ATCC, Rockville, Maryland), which express either NOS2 or β-galactosidase, were cultured in A50 medium (Biofluids, Rockville, Maryland) supplemented with 10% FBS, 1 mM N^G-monomethyl-L-arginine, 5 mM glutamine and 200 μg G418 per ml.

Growth rates were determined by plating cells in triplicate dishes at 10^3 cells per 60-mm dish and staining them with 0.25% crystal violet. The number of cells per colony was determined by counting the stained cells under the microscope. The number of cells was determined in 10 colonies/dish, and population doublings are expressed as log2(cells/colony).

Determination of nitrite plus nitrate. Cells (3×10^6) were plated in 9-mm^2 culture wells (Costar, Cambridge, Massachusetts) and cultured in 4 ml

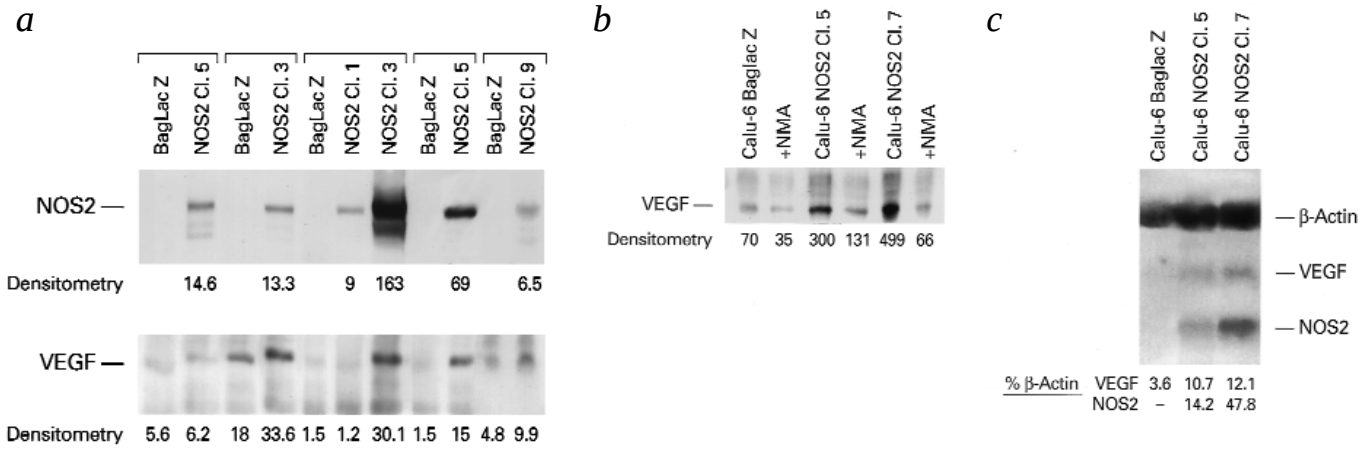

Fig 5 ***a***, Increased VEGF concentration in protein extracts of NOS2-expressing human carcinoma cell lines. Protein extracts were prepared from RKO, HCT-116, HT-29, Calu-6 and LoVo cells infected with the retroviral construct DFG-iNOS. The NOS2 protein band was detected by western blot analysis with a polyclonal anti-human NOS2 antibody and 100 μg of protein extract per lane. NOS2 protein was not found in the vector control cell lines (BaglacZ). VEGF protein concentrations were determined after immunoprecipitation of VEGF using 1 mg of protein extract. Constitutive expression of VEGF in HCT-116 cells has been reported[33]. ***b*** and ***c***, VEGF protein concentration in the culture medium of vector control is 14%–23% its concentration in the culture medium of NOS2-expressing Calu-6 cell clones (*b*); this correlates with their relative VEGF mRNA expression (*c*). Moreover, the NOS inhibitor L-NMA decreases VEGF secretion. Cells (3×10^6) were cultured in 4 ml of medium for 48 hr, with or without 2 mM L-NMA. VEGF was immunoprecipitated from 1 ml of culture medium. The 4.4-kb VEGF mRNA was detected by northern blotting with a 522-bp ^{32}P-labeled cDNA (exons 1–7), and the 7.5-kb polycistronic mRNA encoding NOS2 (ref. 20), with the full-length human NOS2 cDNA (ref. 32).

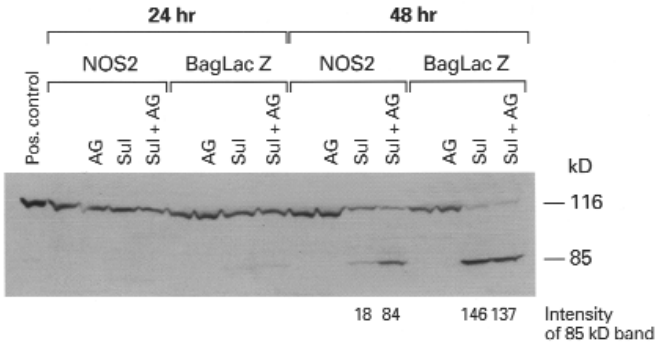

Fig. 6 Nitric oxide inhibits pro-apoptotic PARP cleavage. HT-29 carcinoma cells were treated with 2 mM sulindac (Sul) for 24 or 48 h, and the cleavage of PARP was determined by western blot analysis. The amount of the 85-kDa PARP cleavage fragment is substantially reduced in NOS2-expressing HT-29 cells at 48 h compared with that in vector controls (BagLacZ). Addition of 1 mM AG, an NOS2 inhibitor, increases PARP cleavage by 467% in NOS2-expressing cells, whereas AG does not affect the cleavage of PARP in the BaglacZ cells.

medium for 48 h. To determine nitrite plus nitrate concentrations in culture medium, nitrate was converted to nitrite, and nitrite was determined with the Griess reagent[19].

Tumor xenotransplantation. Suspensions of 3×10^5 to 5×10^6 cells in a volume of 0.2 ml were injected at a single subcutaneous site into athymic nude mice previously irradiated with 350 rads. Either 10 or 20 animals were injected per experiment. A nodule was scored as a tumor when it measured 125 mm^3 or more by its largest two dimensions.

CD31 immunohistochemistry. Sections of ethanol-fixed tumors 5 μm in thickness were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked by treatment with H_2O_2. Sections were incubated with a 1:50 dilution of normal goat serum in PBS/2% BSA and then with the MEC13.3 rat monoclonal anti-mouse CD31 antibody (1:200 dilution; PharMingen, San Diego, California), in PBS/2% BSA for 45 min. Slides were rinsed with PBS and incubated with a secondary, biotin-labeled anti-rat Ig antibody (Vectastain, Vector Laboratories, Burlingame, California). After incubation with an avidin–biotin–peroxidase complex, slides were stained with 3,3-diaminobenzidine for 10–20 min. Microvessels were counted at ×250 magnification (×25 objective and ×10 ocular). At this magnification, eight areas per tumor, not including tumor edges, were scanned, and all CD31-positive vessels were counted.

Western blot analysis. Lysates for western blotting were prepared in RIPA buffer[3]. For analysis of VEGF, 5 μg of rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, California) were added to either 1 mg of cellular protein extract or 1 ml of cell culture medium, incubated for 1 h at 8–10 °C, and then mixed with protein A–sepharose (10 mg) for 1 h. The $p21^{WAF1}$ protein was immunoprecipitated from 100 μg of tumor extract with 10 μg of anti-WAF1 antibody (mAb4; Oncogene Research Products, Cambridge, Massachusetts) and 30 μg of protein G-sepharose. Samples were spun at 10,000*g*, and pellets were washed with RIPA buffer, boiled in SDS/DTT buffer (5,3-Prime, Boulder, Colorado) and loaded on a SDS/13 % polyacrylamide gel. For NOS2 and PARP analysis, 100 μg of soluble protein extract were loaded on a SDS/7 % polyacrylamide gel. After transfer to an Immobilon-P membrane (Millipore, Bedford, Massachusetts), VEGF, NOS2 and PARP proteins were detected with either a polyclonal anti-VEGF antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, California), a polyclonal anti-NOS2 antibody (1:40,000 dilution; Jeffrey Weidner, Merck Research Laboratories), or a polyclonal anti-PARP antibody (5 μg/ml; Upstate Biotechnology, Lake Placid, New York), as described[3]. Levels of $p21^{WAF1}$ protein were determined with a WAF1 western blot detection kit (Ab1; Oncogene Research Products, Cambridge, Massachusetts).

Sulindac-induced apoptosis. HT-29 cells were cultured in DMEM supplemented with 10% FBS in the presence of 2 mM sulindac (Sigma) or treated with HEPES (20 mM) only. After 24 and 48 h, cell lysates were prepared after combining attached and floating cells.

Northern blotting. Total cellular RNA was prepared with the RNeasy™ kit (Qiagen, Valencia, California). RNA (30–50 μg) was resolved on a 1.2% agarose gel containing 6.3% formaldehyde, transferred to a Hybond™-N nylon membrane (Amersham) and hybridized with a ^{32}P-labeled cDNA probe containing either the full-length human NOS2 sequence[32] or 522 bp of the human VEGF sequence common to all known VEGF isoforms. The VEGF cDNA was generated by RT–PCR (Advantage™RT-for-PCR kit; Clontech, Palo Alto, California) using RNA from HCT-116 human colon carcinoma cells. PCR: 32 cycles, 1 min at 58 °C, at 72 °C and at 94 °C using Taq polymerase (Perkin-Elmer, Norwalk, Connecticut) and two cDNA primers (5'–GCCTCCGAAACCATGAACTTTC–3' and 5'–CGAGTCTGTGTTTTTGCAGGAAC–3').

Statistical analysis. The Kaplan-Meier survival analysis was used to calculate the statistical significance of tumor probabilities in different treatment groups. Other comparisons were done by the two-tailed Student's *t*-test. Relationships are considered statistically significant when $P < 0.05$.

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